Title: Effects of Oscillatory Flow on the Nucleation and Crystallisation of Insulin

Authors: Jose V. Parambil, Marc Schaepertoens, Daryl R. Williams and Jerry Y. Y. Heng*

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Abstract

Protein crystallisation has immense potential to be used for separation and formulation applications on an industrial scale. Crystallisation under static condition is often limited by the diffusion of molecules from the bulk solution to the growing crystal surface. This results in a lower overall yield of the product and longer crystallisation periods. Hence, protein crystallisation under flow conditions has attracted attention with the capability to improve the convective protein transport, reducing the crystallisation time and improving yield. In this study, we investigate the effects of flow, specifically intermittent oscillatory flow on protein (insulin) crystallisation. It was observed that the nucleation and crystal yield is very much influenced by the flow conditions and herein we present some key observations in insulin crystallisation conducted under intermittent oscillatory flow. For oscillatory flow velocities ranging from 6 to 16 mm/min at a frequency of 1 cycle/min, a considerable increase in nucleation has been observed with an increase in flow rate resulting in the formation of large number of crystals in tubes with flow compared to the stationary tubes. Also 50% yield for flow crystallisation was achieved in contrast to only 24% yield for the stationary growth over the 48 hour period. The flow condition was thus found to affect the number and size of crystals. In addition, the intermittent flow pattern utilised in this study helps to separately understand the influence of flow on nucleation and crystal growth. The flow strategy proposed herein could potentially be utilised to optimise crystallisation processes for proteins so as to be used in downstream separation and formulation of products.

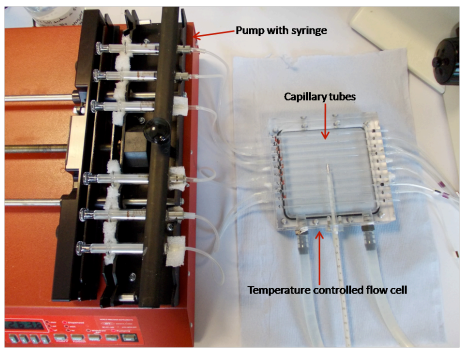

Fig. 1. Flow crystallisation apparatus.

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Keywords: Insulin, protein, flow crystallisation, oscillatory, nucleation, yield

Introduction

Crystallisation has been utilised as a major purification process for small organic molecules for many years now. The pharmaceutical industry uses crystallisation as a means of purification and formulation.[1,2] Similarly, purification of specific proteins and demonstration of their purity was a motive for the development of protein crystallisation in the latter half of the 19th century. Further modifications and development shifted the focus of protein crystallisation as a tool in understanding the properties and the catalytic activity of enzymes. Since the late 1930s, protein crystallisation evolved itself with the primary objective of supplying better crystals for the X-ray diffraction studies[3]. Even at present, this remains one of the most important aspects of protein crystallisation studies around the globe. With the advent of new scientific realms, the application of protein crystallisation is slowly expanding towards biopharmaceutical process designing, formulation of biologics[4] and development of nano-porous media for size exclusion chromatography.[5] However, no comprehensive theory or fundamental database is yet developed that can explain and understand the complexities of protein crystallisation.[3] Most of the difficulties and grey areas in protein crystallisation that was enlisted by Rosenberger[6] in 1986 still continue to persist in this field. Typically, optimisation of the crystallisation conditions is still derived mostly from experimental evidence rather than theoretical approaches.[7,8] In search of answering the complex questions in protein crystallisation, researchers try to explore crystallisation in conjunction with various other facilities and researches such as the use of International Space Station[9] and genomic technologies to create proteins that are easier to crystallise.[10]

Most of the developments in protein crystallisation had focussed on producing high quality crystals from small volumes of protein solution under static condition.[11] Protein crystal growth under quiescent conditions has been reported to be limited by solute diffusion from the bulk of the fluid to the actively growing crystal surface, where the molecules are incorporated into the crystal lattice.[12] This has been demonstrated experimentally and supported by model predictions.[13] Hence, improving the transfer of protein molecules from the bulk to the crystal surface should in theory result in an increase in the rate of crystallisation due to shifting from a diffusion limited to kinetic limited growth regime. A thorough understanding of the underlying mechanism and effects of flow modes on protein crystallisation would be of great use resulting in better yields and control over the process.

With this in mind, flow patterns produced using rotary shakers[14] and alternating electric and magnetic fields[15,16] have been investigated for improving crystallisation in the microscale. Liquid pumps or thermal gradients[17,18] have also been utilised to produce the required flow of crystallising solution. In the crystallisation of chicken egg-white lysozyme (CEWL), Roberts et al. have reported that by using a peristaltic pump configuration to obtain a continuous flow of the protein solution through a crystallising capillary, higher crystal yield, larger crystals and narrower crystal size distribution can be obtained in comparison to stationary crystallisation[19]. Roberts et al. emphasised that protein flow with and without recirculation can enhance crystal growth and is dependent on the flow velocity of the solution through the capillary. Roberts et al. also noted that, the continuous flow in their study has helped only in improving the crystal growth rate and did not appear to affect the nucleation process.

Though the study by Roberts et al. has opened a promising realm in protein crystallisation with increased crystal growth and narrow distribution of crystal size, not every protein would behave like CEWL in a continuous flow pattern. When insulin is used in the flow arrangement as explained by Roberts et al, the, protein denatures within a time span of about half an hour and thereby adversely affected the process. This was not completely unexpected as most proteins are biomolecules that are highly sensitive to shear and other physical strains such as temperature and pressure changes. Also, when working with a protein solution that might crystallise under room temperature, a circulatory flow system might cause the crystallisation of the solute protein throughout the flow channels making it difficult to control the system and produce good quality crystals. Hence, a flow system that can be contained within a confined volume and at the same time being capable of providing the required convective motion is required for encountering these hurdles. Oscillatory motion of liquid within the capillary tubes is a promising solution in this context.

Oscillatory motion of liquid solution for separating the aqueous components based on enhanced diffusion has been studied since late 1960s.[20] Effects of oscillatory flow on crystallisation of organic molecules[21-23] and protein separation using ultra-filtration[24] have also been investigated. But, oscillatory flow mode in capillary has never been reported to be used in protein crystallisation. The flow field produced by oscillatory fluid motion is unique compared to the conditions in a micro-stirring methods with sitting- and hanging-drop vapour diffusion techniques used by Adachi et al.[25], or to the continuous flow used by

Roberts et al.[19], or solution motion induced using electric, magnetic or mechanical methods by other researchers[15,16,26] to study protein crystallisation. The velocity profile produced by oscillatory flow cannot be achieved in most other simple fluid flow systems. It is found from this study that, this uniqueness in the fluid dynamics of oscillatory flow mode can have a profound influence on protein crystallisation that has not been observed in any other methods. Hence, the effect of oscillatory flow mode on protein crystallisation may provide unique opportunities to understand the process and open new perspective on approaching on the bigger problems.

In addition to the oscillatory motion of the protein solution, an intermittent flow patterns is also reported in this study. In intermittent flow study, the crystallising solution is subjected to various duration of flow and static conditions at regular intervals in specific order. This intermittency enables to segregate the effect of the oscillatory flow motion on the nucleation and growth of the protein crystals. As authors' understand, it is for the first time that such an approach have been utilised in studying the effect of flow on protein crystallisation.

This study investigates flow effects on protein crystallisation, focusing on the effect of oscillatory flow on nucleation and crystal growth. This study is a pioneering step in utilising intermittent oscillatory flow in capillaries for understanding and controlling the process of protein crystallisation.

Experimental Methods

Preparation of Insulin Solution

Citrate buffer (0.48 M) at pH 6 was prepared in deionised water using anionic trisodium citrate (Merck) and cationic disodium citrate (BDH Chemicals). The precipitation buffer/precipitant solution was prepared by dissolving Zinc sulfate (Sigma-Aldrich) to a concentration of 1 % (w/v) in a 10 % (v/v) acetone (BDH Chemicals) in 0.48 M pH 6 citrate buffer solution. Stock insulin solution (Sigma-Aldrich, I9278, lot no. 119K8407) containing recombinant human insulin at 5 mg/ml was used in this study. The protein solution was mixed with an equal volume of precipitant solution at room temperature (20-22 °C) to give the crystallising solution with initial concentration of 2.5 mg/ml.

Flow System

A programmable 8 channel syringe pump, *Aladdin-8000* (World Precision Instruments Inc., USA) was used to produce the oscillatory flow in the capillary tubes. 2 ml, 10 ml and 20 ml glass syringes (Sanitex, Switzerland) were used to obtain the required flow velocities. The syringes were connected to the capillary tubes using tygon tubing (SC0021, Ismatech) of 2.06 mm internal diameter. The entire system was set up as shown in Figure 1**.** One oscillation cycle of the syringe pump consisted of a pumping phase and a withdrawing phase. This corresponded to one forward and backward movement of insulin solution inside the capillary tubes. The pump settings and the syringes were adjusted suitably to obtain oscillatory flow of various velocities with a frequency of 1 cycle per minute. The flow velocity of insulin solution was measured by monitoring the movement of the liquid meniscus within the capillary tubes.

Insulin Crystallisation

Crystallisation of insulin was carried out in capillary tubes at 10°C. The soda glass capillaries of 2 mm internal diameter (Bilbate Ltd., UK) were maintained at the desired temperature using a GR 150 digital high performance stirred thermostatic water bath (Grant Instruments Ltd., UK) connected to the crystallisation cell as shown in Figure 1. The insulin solution prepared at room temperature was fed into the capillary tubes after the crystallisation cell was equilibrated at 10°C. This enabled us to maintain the same temperature from beginning till end of the experiment. The crystallisation process was monitored using a reflective microscope (Olympus, BX51M) with a digital camera (Olympus, DP70) connected to a computer with an image capture software *Analysis 5.0* (Soft Imaging Systems).

In the initial set of experiments, only the oscillatory flow velocity of the solution was varied and other factors including temperature, initial protein concentration and the oscillating frequency were maintained constants. Control tubes were maintained at static condition while flow was provided in others. In the latter set of experiments with intermittent flow pattern, the duration of flow and static conditions in the experimental tubes were varied while keeping other factors a constant. In this case, two set of control tubes, one under static condition and other with oscillatory flow throughout the experimental duration were maintained.

Crystal Yield

The crystal yield was measured using modified Bradford's method similar to as described by Bergeron et al.[27] using a UV-Visible Absorption spectrophotometer (Lambda35, PerkinElmer). The supernatant solution from various capillaries are collected into glass vials after 48 hours of crystallisation and mixed with appropriate volumes of Bradford's reagent. 20 minutes of incubation time is allowed prior to the measurement of absorbance at 595 nm. Components in the solution other than the protein were found not to interfere with the measurement of protein concentration using this method. The concentration of protein in the supernatant solution is then found out against the absorbance in standard plot initially prepared with known concentration of protein solutions using the same procedure. Since the protein concentration in the supernatant solution represents the amount of insulin that was not crystallised during the run, this concentration value was then used to calculate the amount of crystals formed and the corresponding crystal yield.

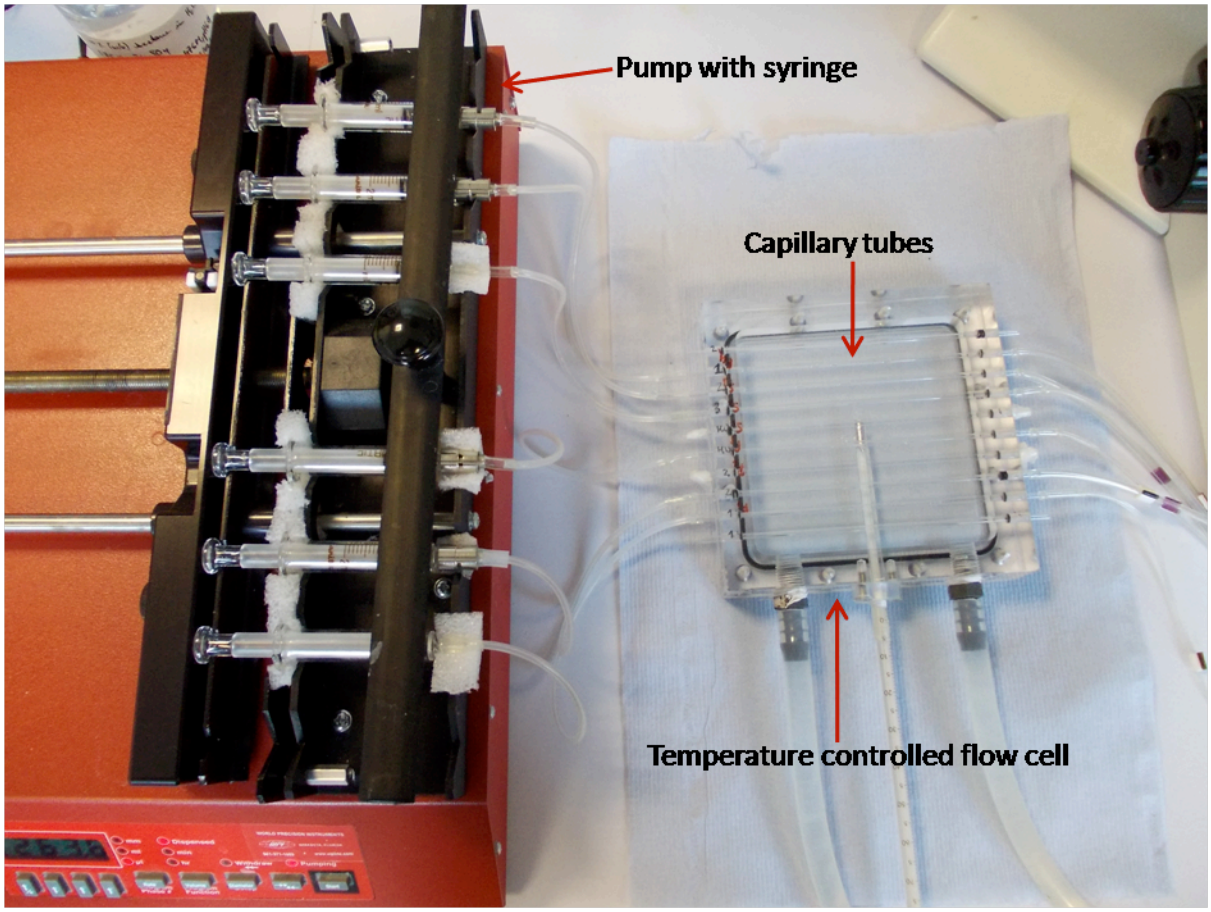

Figure 1: Crystallisation cell and piston pump attached to one of the capillaries for oscillatory flow.

Results

The observations of insulin crystallisation with oscillatory flow could be approached in two different aspects regarding the influence of flow on nucleation and on overall crystal yield. Both these factors are considered in the following discussion.

Effect on Nucleation

Insulin produced a higher number of crystals with flow in comparison to the stationary tubes. The oscillatory flow was found to enhance nucleation in all flow velocities under consideration. Experiments conducted at various flow velocities also found that there exists a strong correlation between crystal nucleation, growth rate and flow rate. Upon increasing the flow rate from 6 mm/min to 16 mm/min, at a constant frequency of 1 cycle per minute, it was observed that the number of crystals and crystal yield increased with increasing flow. Figure 2 gives a representative view on the growth of insulin crystals under various flow rates after 48 hours. As we move from figure 2(i) to 2(v) in the increasing order of oscillating flow velocity, we observe that there is a corresponding increase in the number of crystals. This is an indication that insulin nucleation is enhanced by the oscillatory flow rate at the frequency of 1 cycle per minute within the velocity range of this study, i.e. 6 mm/min to 16 mm/min.

In order to explicitly prove this effect of oscillatory flow on nucleation, delayed-start of flow was utilised. In delayed-start experiments, flow was initiated in different capillary tubes at different times after crystallisation had commenced. That is, crystallisation was started in these tubes under stationary condition, and flow was then provided after specific time delay of 4 hrs (tubes D1 & D2), 8 hrs (tubes D3 & D4) and 12 hrs (tubes D5 & D6). Hence, the crystallisation in these tubes started under stationary conditions, but was then shifted to oscillatory flow conditions with a fixed flow velocity. Two sets of control tubes were maintained with two capillaries each – one set at stationary condition (DS1 & DS2) and another set under permanent oscillation (DF1 & DF2) over the whole course of each experiment. This allowed for segregation of crystallisation behaviour with respect to nucleation and growth pattern of insulin crystals within the scope of the two conditional extremes utilised in this study – no-flow and oscillatory flow throughout.

In the control tubes under stationary condition (DS1 & DS2), the crystals that grew in the 48 hour time period were few in number, but with a size range of 300 to 450 μm. In the control tubes with flow throughout (DF1 & DF2), a large number of crystals was observed and continued to grow for the 48 hour window. The crystal size distribution that was observed in the delayed-start tubes (D1 to D6) was interesting in context of this study. As expected, the crystal growth in the delayed-start tubes remained similar to that within the stationary control tubes until flow was introduced. Once, the flow was provided, a larger number of small crystals were observed growing along with the previously observed crystals. That is, more crystals nucleated after flow was given. This is observed in figure 3. In comparison with DF1 and DF2, the size distribution of crystals in D1 to D6 stands in sharp contrast with a few very large crystals surrounded by a lot number of smaller crystals. This is clearly observable in picture (iv) of figure 3 as there are a large number of crystals of size less than 100 μm alongside a few crystals that are above 200 μm in size. This large size difference is a strong evidence that, the smaller crystals nucleated after flow had been provided while the larger crystals had nucleated and grown during the stationary phase and continued to grow under flow conditions. Thus, we propose that, oscillatory flow has a promoting effect on nucleation of insulin crystals.

Shear rate and Nucleation:

Solution shear flow in protein solution droplets have been reported to increase crystal nucleation.[28] Also, oscillatory shear flow is reported to produce ordered arrangement in colloidal structures with spheres of less than a micrometer in diameter.[29] Even micelles as small as 6 nm have been found to form hexagonal symmetry in macroscopic dimensions under the influence of oscillatory shear flow.[30] The hydrodynamic radius of insulin hexamer has been reported in the range of 5-6 nm.[31] Also, the high density clusters that are suggested to be the precursor of protein crystal nuclei in a two step nucleation process are reported to be in the order of hundreds of nano meters.[32] Hence we suggest that the improvement in the nucleation of insulin crystals could be caused by the shear effect on solution inside the capillary tubes as suggested by Anita et al.[28] and the ordering of the insulin hexamers or the dense liquid clusters occur under the effect of oscillatory flow. Thus the combined effect of the shear and oscillatory fluid motion results in the formation of a large number of crystal nuclei once the flow is initiated. But an extensive

quantification of the nucleation and shear rate in oscillatory flow would be required to find out the optimal shear required for the nucleation of insulin crystals under this condition.

In order to quantify the shear rate developed in this study, fluid flow modelling using COMSOL (Multiphysics Modelling and Simulation Software) was utilised. Using the laminar flow condition in a capillary tube as in the experiment and providing the suitable initial and boundary conditions, the velocity distribution and the shear rate developed within the oscillatory flow was computed. This maximum shear rate for various flow rates used in the study are plotted in figure 4. Also, the shear rate distribution profile within the fluid at the time when this maximum shear rate is experienced in 16mm/min flow velocity is provided in figure 5. The results showed that the shear rate in the fluid varied from $0.01s^{-1}$ to $0.62s^{-1}$. The maximum shear rate increased from $0.23s^{-1}$ for flow velocity of 6 mm/min to $0.62s^{-1}$ for 16mm/min velocity. This shear rate is within the range where ordered structural arrangements of colloidal particles have been observed.[33] This provides further reliability in relating nucleation enhancement of insulin by shear flow to the ordered arrangement of colloids under similar conditions. That is, under the influence of the computed shear rate, the insulin hexamers or high density liquid clusters of insulin forms ordered arrangements in shear planes similar to colloidal arrangement. Once this small scale ordered arrangement is established, the nucleation event could follow rapidly, resulting in large number of nuclei throughout the capillary.

Apart from shear rate, studies on colloidal structures point that the frequency of oscillation could have an impact on the crystallisation behaviour of proteins. Thus, controlling the flow velocity and frequency in an oscillatory flow protein crystallisation would provide us a valuable tool in controlling the event of nucleation and there by resulting in controlled crystallisation as such.

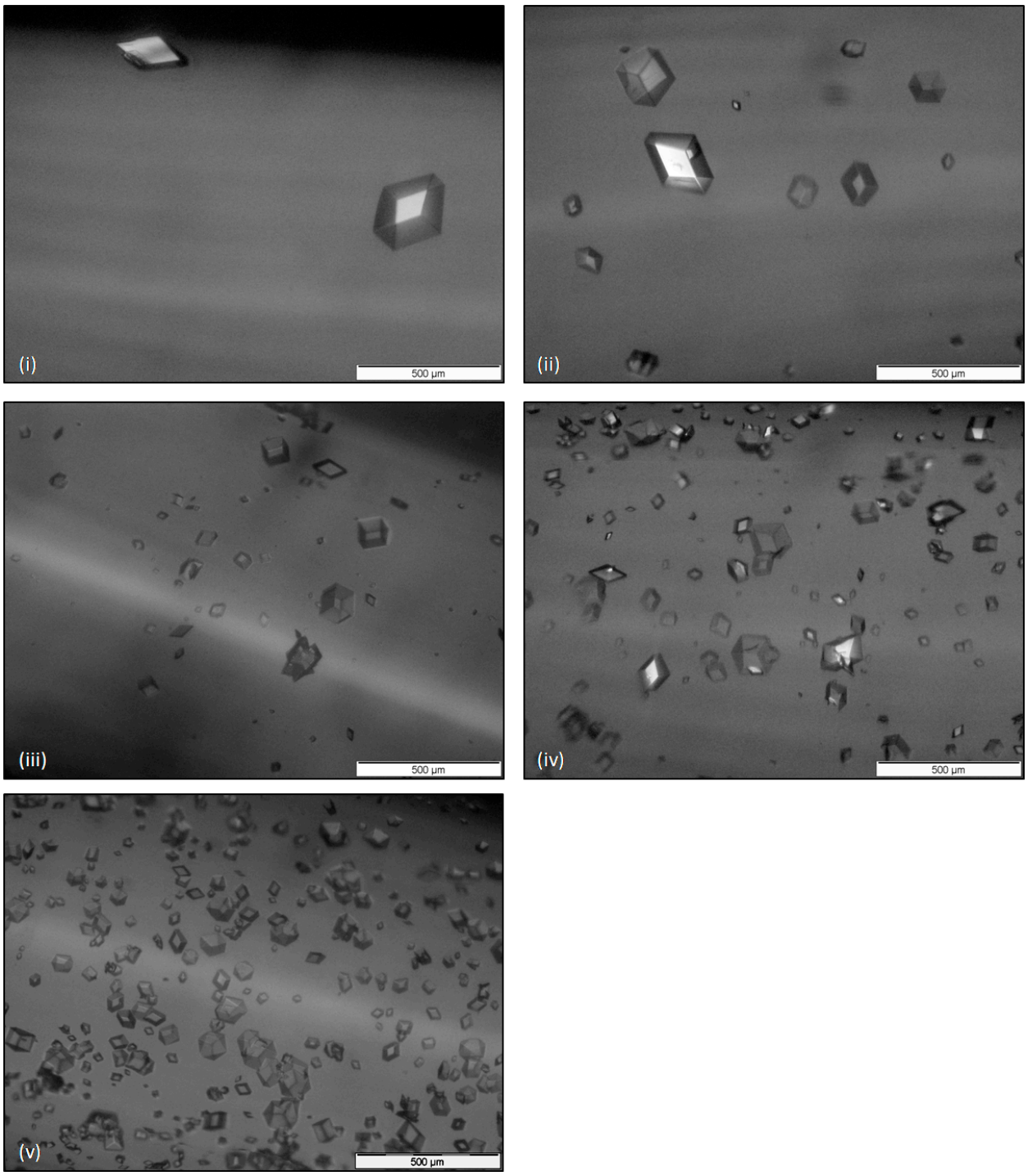

Figure 2: Nucleation enhancement of insulin crystals with flow rate. Pictures after 48 hours in (i) Stationary tube and in capillaries with flow at (ii) 6 mm/min, (iii) 8 mm/min, (iv) 12 mm/min and (v) 16 mm/min

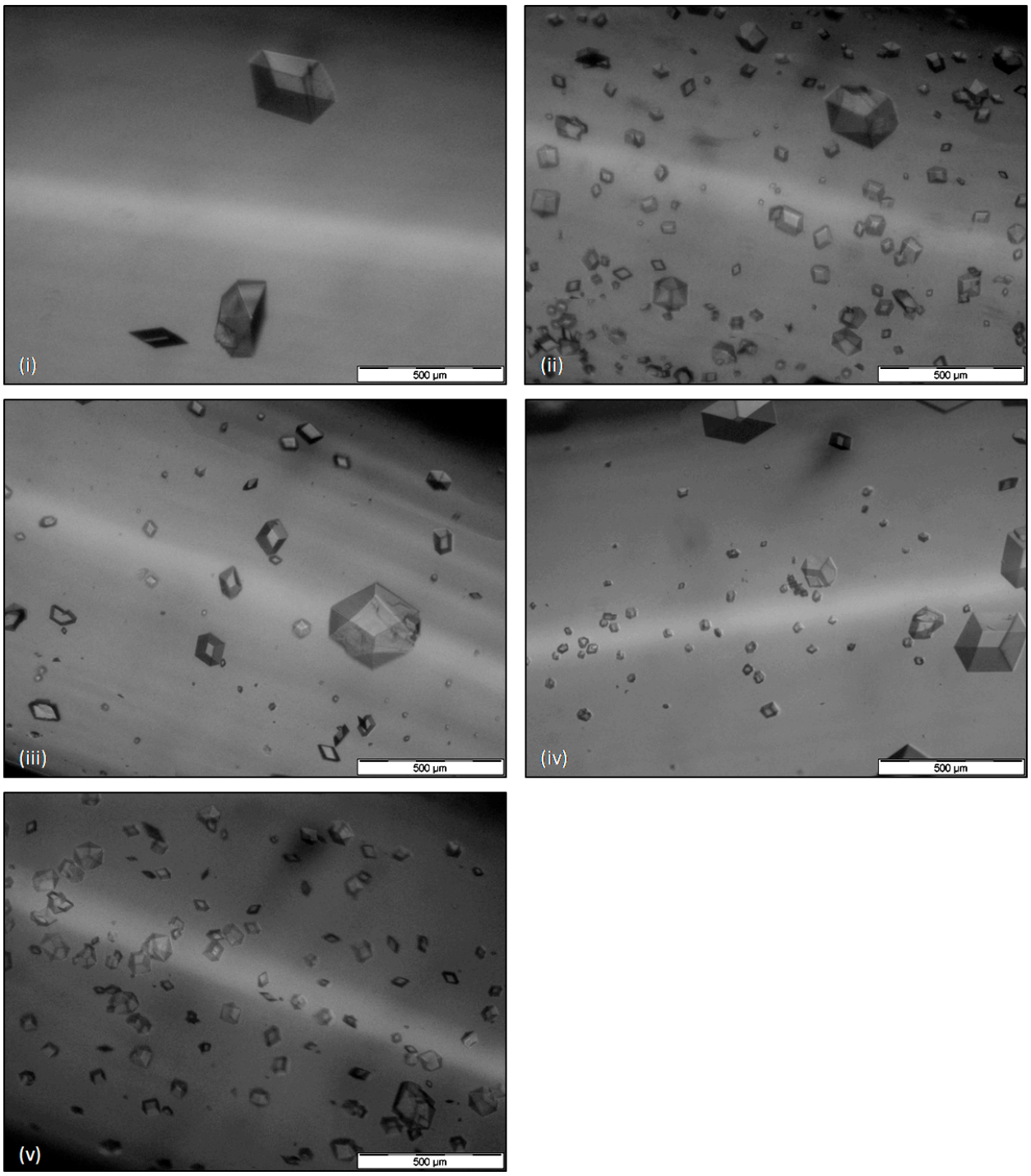

Figure 3: Insulin crystals grown for 36 hours in (i) Stationary Control, DS1 and tubes started under stationary condition and flow initiated after (ii) 4 hours, D1, (iii) 8 hours, D3, (iv) 12 hours, D5 and (v) Flow control, DF1. Flow rate in DF1, D1, D3 and D5 were 12 mm/min at a frequency of 1 cycle per minute

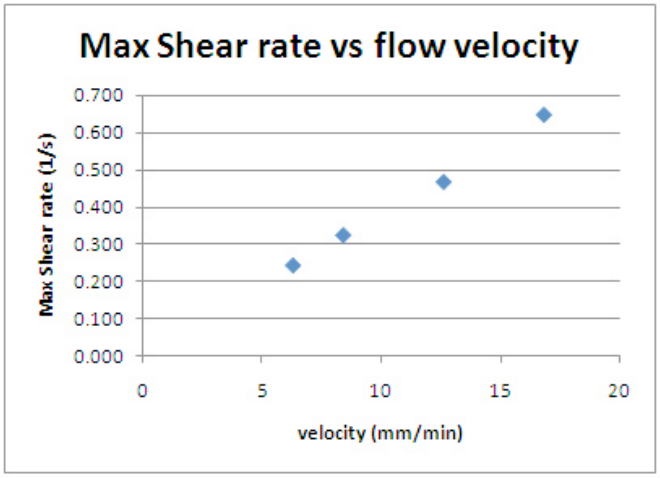

Figure 4: Variation of maximum shear rate vs average flow velocity

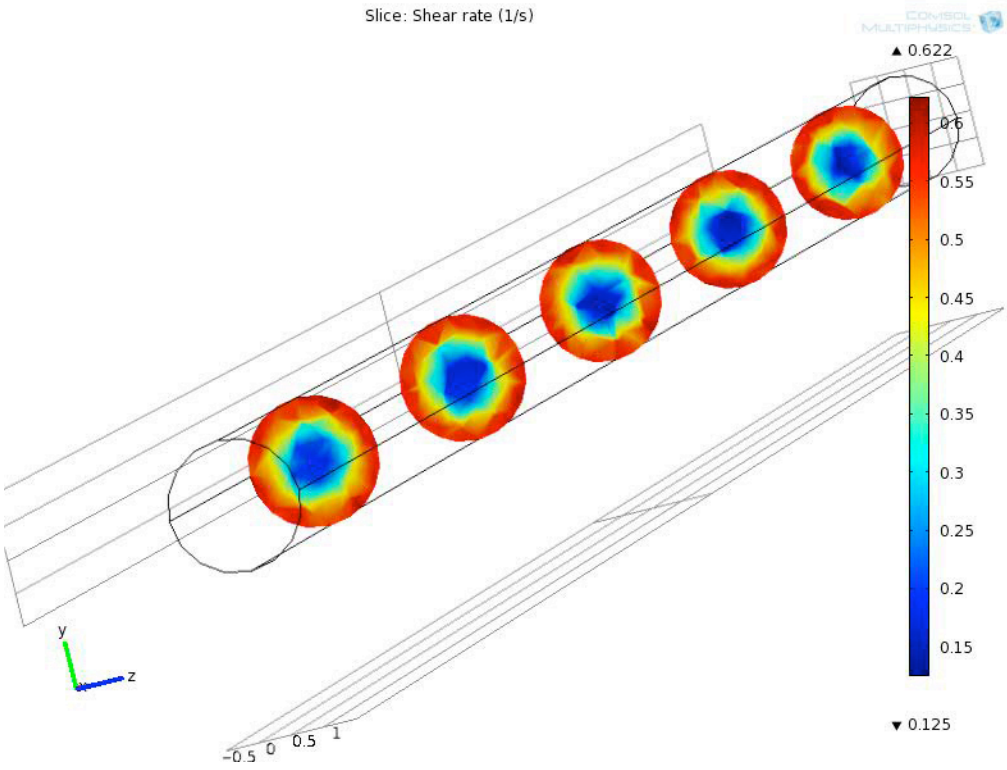

Figure 5: Shear rate distribution profile in fluid with average flow velocity of 16mm/min at the time when the fluid experiences the maximum shear rate

Effect on Crystal Yield

Apart from increasing the number of crystals or obtaining a narrow size distribution of crystals, it is also important to observe efficiency of flow process in recovering protein from solution. It would be useful if we can obtain a comparative analysis on the amount of protein that can be recovered from the crystallising solution between a stationary experiment and flow condition. This will be helpful in determining if the new method would save time and energy in further works. Hence the effect of oscillatory flow on overall crystal yield was investigated and compared with that from stationary crystallisation. As with nucleation, crystal yield studies also exhibits a direct correlation with the flow rate of the crystallisation solution.

The yields for various flow rates are plotted in figure 6. Under stationary conditions for a time period of 48 hours, the insulin crystal yield for our conditions was observed to be 23.5±2.9 %. In case of tubes with oscillations, for the same duration and conditions, the observed yields were in the range of 40–50 %, with a higher yield with increasing flow rate. Thus, a two fold increase of crystal yield can be obtained by providing a flow to the crystallising solution in the range of 6 mm/min to 16 mm/min. It has been reported that at higher flow rates, the effect of impurities in the solution appears to affect the process more than at lower flow velocities[19]. Also, the relative improvement of the effect of flow on mass transfer would be lesser when we compare the increase from static to a low flow rate and from a low flow rate to higher flow rates. Combined with these effects, there would be a finite flow velocity where the crystal yield will be at its maximum and then reduces with increasing flow rate. Clearly, this is above the flow velocity that has been utilised in this study.

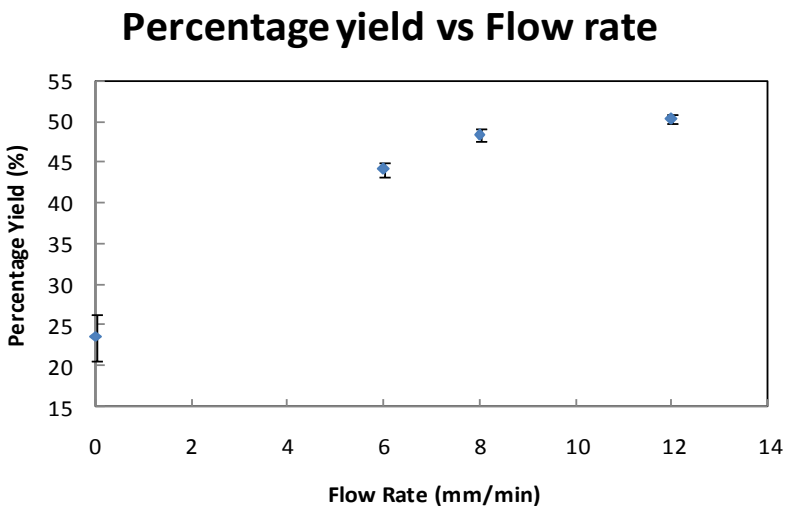

Figure 6: Percentage yield of insulin crystals against flow rate.

Conclusion

We report a methodology for increasing nucleation and yield of insulin crystals using an oscillatory flow in capillary tubes. By utilising the intermittent flow modes, we were able to segregate the effect of oscillatory flow on nucleation and crystal growth. This method has a great advantage over unidirectional flow due to the influence on nucleation behaviour of the protein. Thus, a unique method to control the number of crystals that can be generated in protein crystallisation has been put forward by this study. The influence is clearly not just on the number of the crystals as the crystal yield has increased from 24% under stationary condition to 50% under the highest oscillatory flow rate of 12 mm/min used in this study. Thus the oscillatory flow can be utilised to produce large number of insulin crystals with size less than 50μm while maintaining higher yield of product. This potential has been demonstrated through our study as a number of crystals with about 50μm size are observed in the microscope pictures of crystals growing under oscillatory flow conditions for 48hrs. Since the flow rate and the frequency of the intermittent oscillatory flow can be adjusted independently, it offers a wider opportunity to analyse and control the events of crystallisation. This method is also applicable to a wider spectrum of shear and temperature sensitive proteins that cannot be crystallised under continuous flow modes due to process difficulties and protein denaturation. The flow modes in capillary crystallisation can potentially be optimised to enhance nucleation, increase crystal yields, control of crystal size and potentially be applied for downstream separation and formulation of high value products.

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